Gartenbauwissenschaft, **66** (6). S. 293–297, 2001, ISSN 0016–478X. © Verlag Eugen Ulmer GmbH & Co., Stuttgart

Factors Affecting Adventitious Shoot and Root Formation from the Avocado Cultivar 'Hass' (*Persea americana*, Mill.) *in vitro*

Einflußfaktoren auf die Adventivspross- und Wurzelbildung bei der Avocadosorte 'Hass' (*Persea americana* Mill.) *in vitro*

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Summary

Avocado is an important crop that would benefit from the production of new, superior cultivars; however, conventional breeding techniques for this species are slow. The development of cultivars would be aided if an efficient tissue culture system could be developed. This study describes the use of embryonic material and variations in tissue culture conditions on *in vitro* shoot development. The production of shoot bases from embryonic shoot tissues permitted the production of multiple adventitious shoots from one explant, with up to ten shoots developing from one base. Shoot development was significantly affected by the hormone regime on which the embryonic shoot bases were cultured. MMS medium supplemented with 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3 was found to be optimal for adventitious shoot development. Cutting the embryonic shoot tissue transversely significantly increased multiple shoot growth compared to longitudinal dissection. The addition of 0.1 % PVP reduced tissue browning and necrosis. Root formation was achieved from 75 % of adventitious microcuttings with the use of IBA and PUT, either as a pretreatment or in the culture medium. The combination of these methods should provide an efficient way of achieving multiple, adventitious shoot and root formation *in vitro*.

Zusammenfassung

Avocado ist eine wichtige Frucht. Die Entwicklung neuer, besserer Sorten wäre von großem Nutzen, doch dauert die konventionelle Züchtung dieser Art sehr lange. Ein leistungsfähiges Gewebekultursystem könnte die Entwicklung von Sorten beschleunigen.
Diese Untersuchung beschreibt die Verwendung von embryonalem Gewebe und die Wirkung unterschiedlicher Bedingungen bei der Gewebekultur auf die Sprossbildung *in vitro*. Die Entwicklung von Sprossbasen aus embryonalem Sprossgewebe ermöglicht die Produktion zahlreicher Adventivsprosse aus einem Explantat, mit bis zu zehn Sprossen aus einer Basis. Die Sprossbildung wurde durch die Hormonabfolge der Medien, auf denen die embryonalen Sprossbasen kultiviert wurden, signifikant beeinflußt. MMS Medium mit 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3 ist für die Adventivsprossbildung optimal. Das diagonale Schneiden des embryonalen Sprossgewebes erhöht die Sprossbildung im Vergleich zum Längsschnitt signifikant. Die Zugabe von 0.1 % PVP vermindert das Verbräunen des Gewebes und das Auftreten von Nekrosen. Die Wurzelbildung von 75 % der adventiven Mikrostecklinge wurde durch die Zugabe von IBA und PUT in der Vorbehandlungphase oder in das Kulturmedium erreicht. Die Kombination dieser Methoden stellen einen effizienten Weg zur multiplen Adventivspross- und Wurzelbildung *in vitro* dar.

Introduction

The avocado is a valuable horticultural crop whose consumption would increase considerably if fruit quality, uniformity and seasonal distribution were improved by the development of superior cultivars. Regeneration is the decisive process in most of the *in vitro* manipulation and, unless this process can be obtained consistently, genetic development by somatic methods is not possible. In woody species, regeneration frequencies in tissues of asexual origin are generally low or zero: however, it has been possible to obtain regeneration from apple discs excised from mature tissues (JAMES et al. 1984; WELANDER 1986). An understanding of the factors controlling the regeneration process is, therefore, vital in developing gene transfer and other technologies for crop plants. SCHROEDER (1980) stated that woody species are somewhat more difficult to propagate by tissue culture procedures than herbaceous plants. Thus, the application of *in vitro* techniques to woody species lags far behind those used for other types of plants and cultivar-specific procedures need to be developed for woody species to ensure the success of such techniques.

Tissue culture systems for avocado are not yet fully developed. Shoot tip culture in avocado has been used for the propagation of juvenile material with good success (PLIEGO-ALFARO and BERGH 1992), while attempts to propagate adult material have failed. Adult shoots proliferate poorly in culture (PLIEGO-ALFARO et al. 1987) due in part to apical necrosis and vitrification. Browning and oxidation, which may be linked with ethylene production, have also been identified as problems associated with the tissue culture of avocado (SCHALL, 1987). In contrast to cultures involving adult shoots, callus can be established from almost any avo-

cado explant; however, adventitious bud regeneration has not yet been observed (PLIEGO-ALFARO and BERGH 1992). Occasional success in inducing root development suggests that the *in vitro* culture of avocado is feasible, and this has stimulated continued research. FRIEDMAN et al. (1985) found that polyamines, in combination with auxins, have a regulatory role in the early events of adventitious root formation in mungbean (*Vigna radiata* (L.) R. Wilczek) cuttings.

Micropropagation from mature tissues of the avocado has proven difficult and even with the appropriate choice and pretreatment of avocado explant materials it is difficult to develop a reproducible tissue culture system (AHMED et al. 1997). Therefore, a more feasible way of producing multiple shoots *in vitro* may be to work systematically with embryonic tissues. This paper describes how different pretreatments, concentrations of growth hormones and modes of dissecting embryonic shoots can be utilised to produce multiple adventitious shoots and roots *in vitro* thereby achieving an efficient regeneration system for the avocado cultivar 'Hass'.

Materials and Methods

Plant materials and culture conditions

Mature avocado fruits of the cultivar 'Hass' were obtained from Kulnura, NSW, Australia. The material to be used as explants was washed in running tap water for 10 minutes, surface sterilised by soaking in either 37.5 % (fruit only) or 25 % (seeds) commercial bleach (4 % w/v available chlorine) for 20 min and then rinsed three times in sterile, distilled water before further manipulation. Two media were used for *in vitro* cultures: MS medium (MURASHIGE and SKOOG 1962), and modified MS medium, MMS, comprising MS medium supplemented with 400 mg l^{-1} casein hydrolysate, 0.1 % (w/v) PVP, 50 mg l^{-1} L-glutamine and 50 mg l^{-1} arginine. Both media, which contained 3 % (w/v) sucrose, were adjusted to a pH 5.7 before autoclaving and were solidified with 0.7 % agar. All tissue cultures were maintained at 24 ± 1 °C with a 16 h photoperiod using cool, white, fluorescent light with a photosynthetic photon flux density of 35 mmol m^{-2} s^{-1}.

Hormone pretreatments to induce adventitious shoots

Mature embryos of the cv. 'Hass' were cultured on MS medium until the radicle developed. Aseptic, juvenile, embryonic shoots, were taken as explants and pretreated with MMS liquid medium supplemented with following concentrations of hormones:

(i) 1.0 mg l–1 BAP;
(ii) 2.0 mg l–1 BAP;
(iii) 2.0 mg l–1 BAP + 2.0 mg l–1 NAA;
(iv) 2.0 mg l–1 NAA; and
(v) a hormone-free, control medium.

Twenty explants were incubated on each medium for two days. The explants were then transferred to MS medium without the addition of any growth hormones. The proportion of explants producing callus was recorded two weeks after transfer to MS medium and adventitious shoot growth was recorded six weeks after transfer. To allow further growth to occur, the explants were cultured for an additional four weeks (ten weeks in culture) after which the shoots were excised and subcultured to induce root formation (see below).

Effects of growth hormones in the culture media to induce multiple shoots

In an another experiment, embryonic shoots were taken as explants six weeks after the radicle had emerged and subcultured onto solid MMS medium containing the following concentrations of hormones:

(i) 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3;
(ii) 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} KIN;
(iii) 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA;
(iv) 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} 2,4-D.

Twenty explants were placed on each medium. After four weeks on these media, any adventitious shoots that formed were cut close to their point of formation and the resulting shoot bases were subcultured onto media identical with that used for the initial culture period. This latter procedure was repeated after a further four weeks of culture. Shoot formation was recorded at four weeks from the start of the respective subculture. Any shoots that formed were subcultured to induce root formation.

Excision of embryonic shoot bases

Embryonic tissue of avocado was dissected to give either 2–6 pieces in either: (i) transverse sections or (ii) longitudinal sections. Twenty five sections from each mode of dissection were placed on MMS medium supplemented with 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3.

Root formation from adventitious shoots

To induce root formation, twenty uniformly sized (approx. 20 mm long), aseptic microcuttings were used for each of the following treatments: The different treatments were as follows:

(i) microcuttings were kept directly into RIM without any auxin;
(ii) microcuttings were kept on filter paper bridge for 1 week in 1000 mg l^{-1} IBA and transferred onto RIM;
(iii) microcuttings were dipped in 1000 mg l^{-1} IBA then in 10^{-4} M PUT (10 min each dipping) then transferred onto RIM; and
(iv) microcuttings directly placed on RIM supplemented with 1.0 mg l^{-1} IBA and 10^{-4} M PUT.

Root induction medium (RIM) consisted of WPM supplemented with 0.1 % PVP, 2.0 % sucrose, 0.2 % AC, 0.7 % agar with a pH 5.7. The percentage of roots formed was recorded after 12 weeks of culture. Callus formation was subjectively assessed at the same time using the following scale: none; low (a few patches of callus on the cut face of the microcutting) or moderate (callus surrounding the cut surface of the microcutting).

Table 1. Effect of hormone pretreatments on shoot formation from avocado embryonic shoot of the cv. 'Hass' cultured on MMS medium containing different hormone regimes. The values, transformed to angles before statistical analysis, were derived from twenty explants per treatment. Values followed by the same letter within the columns are not significantly different at the 0.05 or 0.01 probability levels. Where differences do occur they are significant at the p=0.01 level.
Vorbehandlungseffekte auf die Sprossbildung aus embryonalem Gewebe der Avocadosorte 'Hass' durch die Kultur auf MMS Medium mit unterschiedlichen Hormonzugaben. Die vor der statistischen Analyse winkeltransformierten Werte stammen von 20 Explantaten pro Behandlung. Ungleiche Buchstaben innerhalb der Spalten bedeuten signifikante Unterschiede mit einer Irrtumswahrscheinlichleit von p=0.01.

Hormone pretreatment on MMS before subculture to hormone-free MS medium	Percentage of explants producing callus after two weeks	Percentage of explants forming shoots after six weeks
(i) 1.0 mg l^{-1} BAP	0 c	90 a
(ii) 2.0 mg l^{-1} BAP	25 b	60 b
(iii) 2.0 mg l^{-1} BAP + 2.0 mg l^{-1} NAA	75 a	45 bc
(iv) 2.0 mg l^{-1} NAA	40 b	25 c
(v) hormone-free	0 c	0 d

Table 2. Effect of hormones on shoot formation from shoots bases derived from embryonic tissues of avocado cv. 'Hass'. Twenty explants were used for each treatment. Values in parenthesis are the standard error of the mean and means followed by the same letter within the columns are not significantly different at the p=0.05 level.
Wirkung von Hormonen auf die Sprossbildung aus Sprossbasen, die sich aus embryonalem Gewebe der Avocadosorte 'Hass' gebildet haben (20 Explantate pro Behandlung). Die Werte in Klammern sind die Standardfehler der Mittelwerte. Mittelwerte mit gleichen Buchstaben in einer Spalte sind nicht signifikant verschieden auf dem p=0.05 Niveau.

Hormone composition of the media used to induce shoot formation from shoot bases	Mean number of shoots formed during the first subculture	Mean number of shoots formed during the second subculture
(i) 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} IBA + 0.1 mg l^{-1} GA_3	2.8 (0.30) a	4.0 (0.31) a
(ii) 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} KIN	1.6 (0.24) b	2.4 (0.40) b
(iii) 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA	1.2 (0.20) b	1.4 (0.24) bc
(iv) 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} 2,4-D	0.6 (0.24) b	0.8 (0.20) c

Statistical analysis

The data obtained were normalised using angular transformations before being subjected to analyses of variance using COSTAT V4.02, EXCEL V5.0 or ORIGIN V4.1. Least significance differences at the p=0.01 and 0.05 level were determined using the Student-Newman-Keuls range test.

Abbreviations:

AC - activated charcoal; MMS - modified Murashige and Skoog medium; PUT - putrescine; PVP - polyvinylpyrrolidone; RIM - root induction medium; WPM = woody plant medium (LLOYD and MCCOWN 1980).

Results

The formation of callus from embryonic tissue was dependent on the hormone regime and required the presence of at least 2.0 mg l^{-1} BAP or 2.0 mg l^{-1} NAA in the medium (Table 1). Adventitious shoot formation from embryonic shoots could also be induced on basal MS medium; however, this phenomenon was dependent on a hormone pretreatment (Table 1). The highest proportion of explants forming shoots occurred with a pretreatment of 1.0 mg l^{-1} BAP. Increasing the amount of BAP in the medium to 2.0 mg l^{-1} reduced shoot formation. The addition of NAA to the pretreatment medium reduced the ability of BAP to induce shoot formation. However, on its own, NAA was also able to induce adventitious shoot formation though to a lesser extent than BAP. Tissue browning, leading to apical necrosis and tissue breakdown, was a problem during the propagation of microcuttings. Through the inclusion of polyvinylpyrrolidone (PVP) at a concentration of 0.1 % (w/v) to the culture medium this problem was largely overcome.

The hormone regime in the culture and subculture media used for the formation of shoots from shoot bases affected the level of adventitious shoot formation (Table 2). Explants placed on to MMS medium supplemented with 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3 produced the highest average number of shoots with an average of approximately three shoots per explant being achieved during the first subculture period and four during the second subculture.

In an attempt to see if the mode of dissecting embryonic shoot tissue affected the ability to form adventitious shoots, embryonic axes were cut into either longitudinal or transverse sections with respect to the axis of growth. It was found that cutting the explant transversely significantly increased the ability of explants to form multiple shoots (p=0.01) with explants cut in a transverse mode producing over three times the number of shoots as those made in the longitudinal mode (Table 3). Dissecting the explants in the transverse mode gave an average of four adventitious multiple shoots per explant after six weeks on MMS medium supplemented with 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3 and an average of ten adventitious multiple shoots after twelve weeks in the same medium. In contrast, the longitudinal mode of dissection only resulted in an average of one shoot per explant after six weeks and four shoots after twelve weeks on the same medium.

Table 3. Effect of mode of dissecting embryonic tissue on the formation of adventitious shoot of avocado cv. 'Hass' *in vitro*. Twenty-five explants were used in each treatment. The dissecting explants were cultured on MMS medium supplemented with 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3. Values in parenthesis are the standard error of the mean and values followed by the differing letters are significantly different at the 0.01 probability level.
Wirkung der Schnittmethode bei embryonalem Gewebe auf die in vitro Sprossbildung der Avocadosorte 'Hass' (25 Explantate pro Behandlung). Die zerteilten Explante wurden auf MMS Medium mit 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3 kultiviert. Die Werte in Klammern sind die Standardfehler der Mittelwerte. Werte mit ungleichen Buchstaben in einer Spalte sind signifikant verschieden auf dem p=0.05 Niveau.

Mode of dissection	Mean number of shoot formation after six weeks in culture	Mean number of shoot formation after twelve weeks in culture
Longitudinal	1.36 (0.09) b	3.24 (0.13) b
Transverse	4.04 (0.14) a	10.00 (0.49) a

IBA in the presence of the diamine putrescine was most effective in promoting root formation in avocado. Both pretreatment with 1000 mg l^{-1} IBA and 10^{-4} M PUT for ten min each before transfer to supplemented WPM and direct transfer of explants to supplemented WPM containing IBA and PUT were the most effective treatments promoting root initials from microcuttings. However, those given the pulse treatment did not produce as much callus. Those microcuttings kept on a filter paper bridge for one week in 1000 mg l^{-1} IBA produced root primordial during this stage, but few developed into fully formed roots after twelve weeks on RIM. No root initials were evident in the treatment where microcuttings were cultured without pretreatment or hormones.

Discussion

The objectives of this study were to find the optimal culture conditions for the induction of multiple adventitious shoots from juvenile avocado tissue. A number of studies (e.g. PLIEGO-ALFARO et al. 1987; KANTHARAJAH et al. 1992) have shown that juvenile tissues can be induced to regenerate where similar attempts using mature tissues have failed. Therefore, emphasis was placed on the use of juvenile tissues in this study. We have shown that embryonic tissue is a suitable source of explants for producing adventitious shoots. However, tissue browning, apical necrosis and tissue breakdown was initially a problem in this study but was overcome through the inclusion of 0.1 % (w/v) PVP to the culture medium. PVP binds phenolic compounds and was essential to the success of the tissue cultures. Similar beneficial effects of PVP have also been reported by HARTY (1985), SCHALL (1987), DALSASO and GUEVARA (1989) for *Persea* spp.

The method of culturing embryonic shoot tissue greatly influences the number of shoots that can be obtained from one embryo. Central to the production of multiple shoots is the establishment of an embryonic shoot base. To do this the first flush of shoots produced from the embryo is cut close to their point of formation from the explant. This induces the production of further shoots and, using this technique, it has been possible to produce up to ten shoots from one shoot base during a single subculture. The efficiency of shoot production can be increased by the formation of multiple shoot bases from a single embryo. The mode of dissection of the embryonic shoot tissues greatly influences the success of this procedure. Cutting the embryonic shoot transversely to the axis of growth induced a higher number of shoots from each section than by making the cuts in a longitudinal manner. It is possible that a greater number of cambial cells are exposed when the embryonic shoots are cut in a transverse manner and this leads to the higher number of shoots that are formed. The composition of the culture medium also affected the frequency of shoot formation from embryonic shoot bases. Of the treatments applied to the shoot bases, the inclusion of 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3 into the culture medium resulted in the highest average number of shoots produced per embryonic shoot base. KANTHARAJAH et al. (1992) also found that the embryonic tissues pretreated in BAP solution started to elongate and the emerged radicle developed further in lychee. RASAI et al. (1994) also indicated that cytokinin was essential for the induction of adventitious buds from *Atemoya*. In this study, the inclusion of 0.1 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3 in addition to 1.0 mg l^{-1} BAP in culture media increased multiple adventitious shoot formation from embryonic shoots. Therefore, a mixture of different types of hormones are required to induce shoot formation and variations in both the types and concentrations of these three hormone groups may increase shoot formation further.

The maximum number of roots produced from single microcuttings was obtained from treatments with both PUT and IBA; however, less callus was formed when these compounds were used as a pretreatment. These results suggest that root formation is resulting from an increase in cell division caused by PUT, plus the influence of IBA on differentiation and cell enlargement. FRIEDMAN et al. (1985) also reported similar ideas from studies of adventitious root induction in mungbean. It

Table 4. Effect of pretreatment and growth hormones on root formation from adventitious microcuttings of avocado cv. 'Hass'. Values followed by the same letter within the columns are not significantly different at the p=0.05 level.
Wirkung der Vorbehandlung und von Wachstumshormongaben auf die Wurzelbildung von adventiven Mikrostecklingen der Avocadosorte 'Hass'. Mittelwerte mit gleichen Buchstaben in einer Spalte sind nicht signifikant verschieden auf dem p=0.05 Niveau.

Treatments	% microcutting forming roots	Callus formation
T1 (control – explants placed directly on RIM).	Nil (c)	None
T2 (pretreated on filter paper bridge soaked in IBA).	55 (b)	Moderate
T3 (pretreated with a pulse of IBA + PUT).	75 (a)	Low
T4 (Directly placed on RIM with IBA + PUT).	60 (ab)	Moderate

is also possible that PUT might act synergistically to enhance increased rooting percentage through interactions with rooting inhibitors or promoters such as peroxidases, cytochrome oxidase or succinic dehydrogenase (FRIEDMAN et al. 1985). It also appears that the age and quality of microcuttings affects their ability to form roots. Observations made during this current study suggest that more juvenile microcuttings and those with larger leaves produced roots more quickly than more mature microcuttings and those with smaller leaves. PLIEGO-ALFARO (1992) also reported similar results in avocado. Therefore, the interactions between shoot, formation, root formation and the culture conditions need much further study.

Avocado plants are protogynous, exhibit synchronous dichogamy and usually require a pollinator plant to achieve fruit set (NIRODY 1922; STOUT 1923). Therefore, embryos and hypocotyls and the plants that regenerate from them are hybrids. As a result, true-to-type material is not produced which could be a problem for the mass multiplication of plants for plantations. However, the tissue culture techniques described in this study would permit the multiplication of hybrid varieties where directed crosses have been made. Also, although the breeding system of avocado is designed to favour cross-pollination, several studies have shown that self-pollinations are possible and can result in significant fruit set (DAVENPORT et al. 1994; KOBAYASHI et al. 2000). Regeneration from shoot tips and axillary buds of a clonal tissue does not suffer from the problem associated with hybridity. Therefore, the establishment of shoot bases, as produced in this study, but derived from these sources, in combination with the appropriate culture conditions, could provide an efficient means of obtaining clonal material. This would speed the development and multiplication of new avocado cultivars via tissue culture.

This project was partially funded by UWS internal research grant.

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Eingereicht: 13.07.01/ 25.09.01

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